

# Effects of Storage and Handling Conditions on Concentrations of Individual Carotenoids, Retinol, and Tocopherol in Plasma

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We investigated the effects of storage and handling on measured values for carotenoids, retinol, and tocopherol in plasma. We found no significant differences in the concentrations of these analytes measured in plasma samples that were frozen immediately after separation as compared with replicate samples maintained at room temperature in the dark for 24 h. Analytes were stable in solvents for at least 18 h at 23 °C after extraction. Purging samples with nitrogen gas before freezing had no detectable beneficial effects. All analytes were stable in plasma stored at -70 °C for at least 28 months or at -20 °C for five months. By 15 months the concentrations of carotenoids were significantly less ( $P < 0.05$ ) in plasma stored at -20 °C than in plasma stored at -70 °C, while retinol and tocopherol concentrations were not significantly different. Concomitant with the decrease in carotenoids was the appearance of unidentified peaks in the ultraviolet. Adding ascorbic acid or butylated hydroxytoluene antioxidants to the precipitating solvent did not alter the losses of carotenoids or alter the appearance of unidentified peaks. Under appropriate conditions, plasma carotenoids, retinol, and tocopherol are stable for more than two years.

Recent interest in the role of nutrients such as carotenoids, retinoids, and tocopherol in cancer prevention has provided impetus for the development of new methods for the separation and quantification of these fat-soluble compounds from biological samples (1-10). However, controversy exists concerning the stability of carotenoids, retinol, and tocopherol (9-16) in such samples. For example, Driskell et al. (11) initially reported that retinol and tocopherol are stable in sera stored at -20 °C for five to eight years. The same group later reported that losses of these analytes upon storage were prevented by addition of ascorbic acid (12). Others have reported significant losses (9), increases (13), and no change (14) in the concentrations of tocopherol and retinol when stored at -20 °C or higher. Several reports (6, 9, 13-15) agree that total carotenoids in serum rapidly decline when stored at temperatures of -20 °C and higher; however, there is little information regarding the stability of individual carotenoids in plasma or serum.

Our purpose here was to examine the effects of various storage and handling conditions on the measured concentrations of carotenoids, retinol, and tocopherol in plasma, to assist in the planning of clinical trials and epidemiological studies.

## Materials and Methods

**Reagents.** Crystalline alpha-carotene, beta-carotene, lycopene (Sigma Chemical Co., St. Louis, MO), zeaxanthin, cryptoxanthin, and echinenone (Hoffmann-La Roche, Nutley, NJ) were used as standards for carotenoid analysis. Retinol, alpha-tocopherol, and alpha-tocopherol acetate (Sigma) were used as standards for retinol/tocopherol assay. We used the following "HPLC grade" reagents: "reagent" alcohol (ethanol, methanol, isopropanol, 95/5/5 by vol), methanol, acetonitrile, hexane, methylene chloride, petroleum ether (Fisher Scientific, Silver Spring, MD), and acetone (J. T. Baker Chemical Co., Phillipsburg, NJ).

**Blood samples.** Blood was sampled from the antecubital vein with "Minicath" needles (Deseret Medical, Sandy, UT) and all-plastic "Monovet" syringes (Sarstedt, Princeton, NJ) containing 4.5 USP units of sodium heparin per milliliter of blood. After centrifugation at 12 °C in subdued light at 2260 × *g* for 20 min, aliquots of plasma were transferred to 1.8-mL "Cryotubes" (Thomas Scientific, Swedesboro, NJ).

**Standard solutions and controls.** Approximately 1 mg of each crystalline carotenoid was dissolved in 2 mL of hexane and diluted to 50 mL with reagent alcohol. Each standard solution was filtered through a 0.5-μm (pore size) filter (Millipore, Bedford, MA) into a red glass volumetric flask. Alpha-tocopherol and alpha-tocopherol acetate were dissolved directly in reagent alcohol. Crystalline retinol was dissolved in 1 mL of acetone, then diluted to 50 mL with petroleum ether. This solution was purified on a 1 × 30 cm column packed with 200-mesh alumina (Fisher Scientific) previously 10% hydrated (vol/wt) and equilibrated with petroleum ether. The less-polar contaminants were eluted from the column with acetone/petroleum ether (2/98 by vol), retinol was eluted with acetone/petroleum ether (10/90 by vol). This eluate was dried under nitrogen and redissolved in hexane. Stock standards were made by diluting the hexane solution with reagent alcohol. To determine concentration of each standard solution, we measured the absorbance at the wavelength indicated, then divided by the appropriate absorptivity as follows:

Compound	Wavelength, nm	Absorptivity, E <sub>1</sub> <sup>1</sup> % <sub>cm</sub>
Zeaxanthin	452	2350
Cryptoxanthin	452	2370
Echinenone	462	2160
Lycopene	472	3450
Alpha-carotene	444	2800
Beta-carotene	453	2590
Retinol	325	1780
Alpha-tocopherol	292	75.8
Alpha-tocopherol acetate	285	43.6

As assessed by "high-performance" liquid chromatography (HPLC), the purity of the standards was at least 90%. We adjusted the concentration of the standards used to prepare standard curves by this percentage. Prepared as indicated above, the internal standards, alpha-tocopherol

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acetate and echinenone, were found to be stable for at least six months when stored at 4 °C in reagent alcohol. A sample of pooled plasma with an established chromatographic profile was analyzed three times per day, to monitor chromatographic performance and provide quality control.

**Sample preparation.** Frozen plasma samples were thawed, mixed, and centrifuged at  $1040 \times g$  for 5 min to remove fibrous debris. We added 150  $\mu\text{L}$  of a mixture of internal standards (echinenone and alpha-tocopherol acetate) to 150  $\mu\text{L}$  of plasma and vortex-mixed for 30 s. Then we added 300  $\mu\text{L}$  of hexane to this mixture and the contents of the tubes were vortex-mixed for 45 s. Precipitated proteins were removed by centrifugation ( $1040 \times g$ , 5 min) at room temperature. Duplicate 100- $\mu\text{L}$  aliquots of the organic phase were removed from each sample and the solvent was evaporated under nitrogen. One aliquot from each sample was dissolved in 100  $\mu\text{L}$  of acetonitrile/methylene chloride/methanol (70:20:10 by vol), the other in 100  $\mu\text{L}$  of reagent alcohol, and both were vortex-mixed for 30 s before analysis for carotenoids or retinol/tocopherol, respectively.

**Chromatographic system.** A single gradient-type HPLC system was converted into two isocratic systems for the parallel analysis of plasma carotenoids and retinol/tocopherol. The HPLC systems consisted of two solvent-delivery systems (Beckman Model 114M), two detectors (Beckman Model 160 UV/VIS) equipped with a 436-nm and a 280-nm filter, a Beckman 450 Data System/Controller, an Altex Model 270A injector (Beckman Instruments, Inc., San Ramon, CA), and a Rheodyne Model 7125 injector (Rainin Instrument Co., Woburn, MA). The columns were either a Supelcosil C<sub>18</sub> 5- $\mu\text{m}$  (250  $\times$  4.6 mm) paired with a matching guard column (Supelco, Bellefonte, PA) or a 250  $\times$  4.6 mm Altex Ultrasphere C<sub>18</sub>, 5- $\mu\text{m}$ , paired with an NH<sub>2</sub> guard column (Alltech, Deerfield, IL). The latter combination provided optimal resolution of the carotenoids, especially the xanthophylls.

The mobile phase for retinol/tocopherol analysis consisted of 100% methanol; that for analysis of carotenoids was acetonitrile/methylene chloride/methanol (70:20:10 by vol). The flow rate for both systems was 1.8 mL/min and run time was less than 12 min. Detector sensitivity for both systems was 0.005 A full scale. Solvents were freshly prepared and de-gassed by reduced-pressure filtering through a 0.5- $\mu\text{m}$  filter.

**Design.** Half of the vials containing plasma were purged

with nitrogen, the other half were not. Some samples from each set were frozen and immediately stored at either -70 °C or -20 °C. Remaining samples were maintained at room temperature (22–24 °C) in the dark for either 4, 12, or 24 h before being frozen and stored at -70 °C. Samples were analyzed intermittently during a 28-month period.

**Calculations.** Initially, standard curves were established by using known concentrations of carotenoids, retinol, and alpha-tocopherol. Response factors for analytes were generated based upon the peak-area ratios vs peak-concentration ratios, with echinenone used as an internal standard for carotenoids and alpha-tocopherol acetate as an internal standard for retinol/tocopherol. These response factors were maintained through the course of the study. If the analytes measured in a control plasma sample varied by more than two standard deviations from their established means, response factors were adjusted to account for variances in column performance. The nomenclature used here for the plasma carotenoids was that of Bieri et al. (1). The response factor for the "unknown" peak was assumed to be 1.0 and that for "precryptoxanthin" was assumed to be the same as cryptoxanthin. For stored samples of plasma, we determined the concentration of analytes by peak area, using the appropriate internal standard. Each chromatogram was visually reviewed to ensure correct baseline position and integration.

**Statistics.** Regression analysis was used to determine whether vitamin concentrations changed as a function of time. When comparisons were made between treatment groups, differences were determined by using the General Linear Models Procedure and the Student–Newman–Keuls Test (17). The level of significance was  $P < 0.05$ .

## Results

**Effect of nitrogen purge on analyte stability.** Analysis after various intervals was performed with and without nitrogen purge. Under the conditions used for the storage of these plasma samples, we saw no beneficial effect of purging with nitrogen. Table 1 presents representative data obtained from samples analyzed after three months of storage.

**Stability of analytes in plasma at room temperature.** The concentrations of total carotenoids, retinol, and tocopherol were similar ( $P > 0.05$ ) in samples of plasma frozen immediately and those that were maintained at room temperature for up to 24 h after harvest, then frozen (Table 1). Samples

**Table 1. Effect of Duration at Room Temperature and Nitrogen Purge on Vitamin and Carotenoid Stability in Plasma<sup>a</sup>**

Group	n	Retinol	Tocopherol	L/Z <sup>b</sup>	U	PC	CRYPT	LYC	A-C	B-C	Total
		Concn, mg/L									
0 h	4	59.2 <sup>c</sup>	970	30.4	10.7	7.7	8.7	59.5	5.1	30.7	152.8
		3.6	75	0.8	0.8	0.7	0.6	2.7	0.5	0.9	5.4
4 h	4	57.6	967	29.6	10.9	7.6	8.4	56.7	4.9	29.8	147.9
		2.7	39	0.5	1.1	0.6	0.4	3.3	0.3	0.1	7.3
12 h	4	59.2	906	30.0	10.7	7.9	8.4	56.8	5.0	30.1	148.9
		2.6	46	2.0	0.2	0.2	0.3	3.2	0.3	1.4	5.2
24 h	4	59.2	911	29.1	10.9	7.8	8.2	56.0	4.7	31.1	147.8
		3.4	40	0.7	0.5	0.5	0.6	3.0	0.8	2.3	6.4
+N <sub>2</sub>	8	58.8	920	30.0	10.4	7.6	8.4	56.3	5.0	30.0	147.7
		3.1	46	1.1	0.5	0.3	0.3	2.5	0.1	0.8	2.2
-N <sub>2</sub>	8	58.7	954	29.6	11.1	7.9	8.4	58.1	4.8	30.9	150.8
		1.8	41	0.5	0.7	0.2	0.2	1.0	0.3	1.1	5.0

<sup>a</sup> Samples stored at -70 °C for three months before assay.

<sup>b</sup> Carotenoids are designated L/Z, lutein/zeaxanthin; U, unknown; PC, pre-cryptoxanthin; CRYPT, cryptoxanthin; LYC, lycopene; A-C, alpha-carotene; B-C, beta-carotene; Total, total carotenoids.

<sup>c</sup> Mean  $\pm$  standard deviation.

were analyzed at the same time, to minimize variation, and results were not different from samples analyzed before freezing.

**Stability of analytes in organic solvents.** To determine whether overnight automated analysis was feasible, we examined the stability of extracted analytes in HPLC solvent at ambient temperature. Plasma extracts were analyzed at once and 18 h after extraction. There was no significant ( $P > 0.05$ ) alteration in the concentrations of retinol, tocopherol, or individual carotenoids after 18 h in solvent at room temperature in the dark (Table 2).

**Effect of duration of storage on analyte stability.** We maintained plasma samples at  $-70^{\circ}\text{C}$ , to determine whether storage duration influenced vitamin stability. Using regression analysis, we saw no correlation between storage time and measured retinol, tocopherol, or carotenoid concentrations during a 28-month period (Figures 1, 2 and 3, respectively).

**Effect of storage temperature on analyte stability.** Plasma samples stored at  $-20^{\circ}\text{C}$  were occasionally analyzed in conjunction with plasma samples stored at  $-70^{\circ}\text{C}$ . For as long as five months, we could see no significant difference ( $P > 0.05$ ) in analytes in plasma stored at  $-20^{\circ}\text{C}$  compared with  $-70^{\circ}\text{C}$  (Figure 4). However, we saw a significant ( $P < 0.05$ ) decrease in the concentration of the carotenoids when we compared samples stored for 15 months at  $-20^{\circ}\text{C}$  with samples at  $-70^{\circ}\text{C}$  (Figure 4). The concentrations of lutein/zeaxanthin, lycopene, alpha-carotene, and beta-carotene were 24, 51, 10, and 26% lower, respectively, in samples stored for 15 months at  $-20^{\circ}\text{C}$  compared with samples stored at  $-70^{\circ}\text{C}$ . In contrast, there was no significant change in retinol or tocopherol concentration between these samples.

Unexpected broad peaks of  $A_{280}$ -absorbing species with retention times of 12–18 min appeared in plasma samples stored at  $-20^{\circ}\text{C}$  for 15 months (Figure 5). The addition of either 1 g of ascorbic acid or 250 mg of butylated hydroxytoluene per liter as antioxidants to the reagent alcohol used to precipitate the plasma proteins did not prevent the appearance of these peaks after long-term storage at  $-20^{\circ}\text{C}$ . Similarly, the measured concentrations of retinol, tocopherol, and carotenoids were not altered by the presence of these antioxidants.

## Discussion

The method described here represents a modification of the methods of Bieri et al. (1, 2) for the parallel assay of plasma retinol, tocopherol, and seven carotenoids. By the addition of a column and injector, a gradient-type HPLC system was converted to two separate isocratic systems, operating simultaneously. This alteration reduced the amount of sample handling and decreased variability in the analysis of any given sample. This technique is particularly

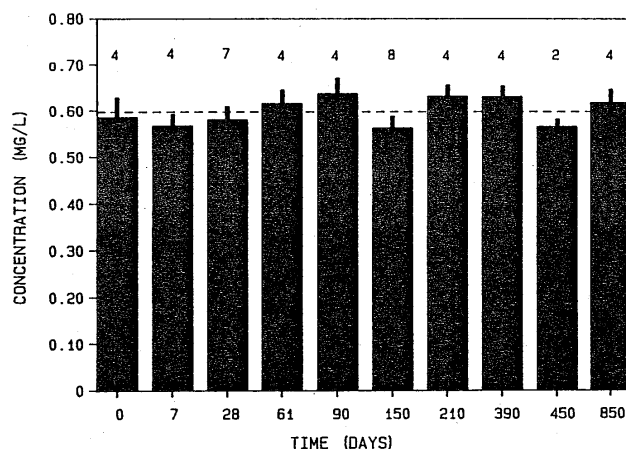


Fig. 1. Effect on measured concentration of retinol of storing plasma at  $-70^{\circ}\text{C}$  for as long as 28 months

The dashed line represents the mean for the 28-month period. Numbers above the bars indicate n determinations at that time point. The lines topping the bars are equivalent to 1 SD

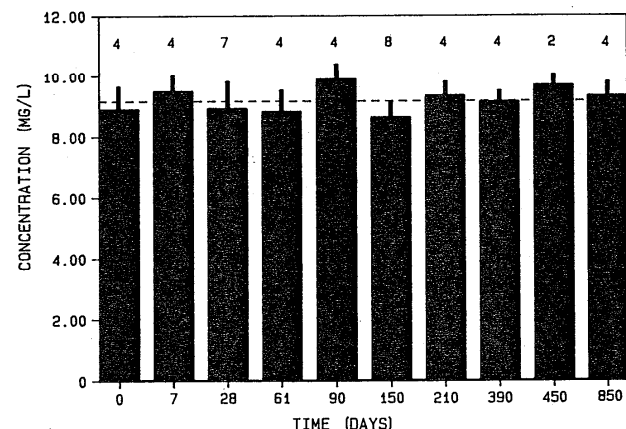


Fig. 2. Effect on measured concentration of tocopherol of storing plasma at  $-70^{\circ}\text{C}$  for as long as 28 months

The dashed line represents the mean for the 28-month period. Numbers above the bars indicate n determinations at that time point. The lines topping the bars are equivalent to 1 SD

useful for clinical trials and screening because it is rapid, sensitive, and reproducible. Our day-to-day coefficients of variation ranged from 4% for retinol to 10% for alpha-carotene, and analytical recovery ranged from 94% to 100% for all analytes. Although adaptable for the simultaneous determination of these vitamers with a single instrument and monitoring two or more wavelengths, our efforts to establish such a system have resulted in decreased resolution, increased analysis time, and (or) spectral interference resulting in poor quantification.

Table 2. Stability of Vitamins and Carotenoids Extracted into Organic Solvents<sup>a</sup>

Time	n	Retinol	Tocopherol	L/Z <sup>b</sup>	U	PC	CRYPT	LYC	A-C	B-C	Total
		Concn, mg/L									
0 h	3	61.2 <sup>c</sup>	1114	26.0	12.1	5.2	9.2	44.1	6.7	47.1	150.4
		1.0	72	0.6	0.3	0.3	0.2	2.5	0.2	1.4	4.7
		2.2	36	0.5	0.8	0.2	0.4	4.7	0.4	4.6	10.8
18 h	3	62.3	1076	26.5	12.1	5.1	8.6	44.4	6.6	46.1	149.4

<sup>a</sup>For this experiment a different plasma sample was used, which contained similar vitamin concentrations as that used for all other experiments.

<sup>b</sup>Abbreviations as in Table 1.

<sup>c</sup>Mean  $\pm$  standard deviation.

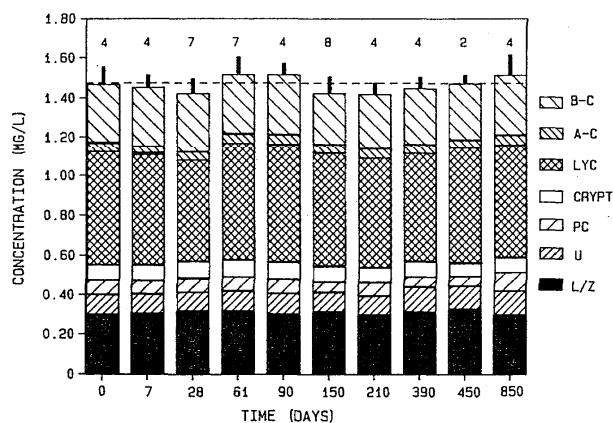


Fig. 3. Effect on measured concentration of carotenoids of storing plasma at  $-70^{\circ}\text{C}$  for as long as 28 months

The dashed line represents the mean over the 28-month period. Numbers above the bars indicate  $n$  determinations at that time point. The lines topping the bars are equivalent to 1 SD. Carotenoids are designated B-C, beta-carotene; A-C, alpha-carotene; LYC, lycopene; CRYPT, cryptoxanthin; PC, pre-cryptoxanthin; U, unknown; and L/Z, lutein/zeaxanthin

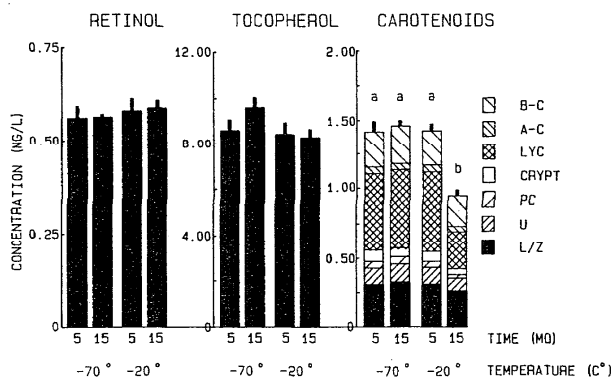


Fig. 4. Comparison of carotenoid, retinol, and tocopherol concentrations in plasma stored at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$  for five and fifteen months. Lines topping the bars are equivalent to 1 SD. Carotenoid designation as in Figure 3. Bars not sharing the same letter are different at  $P < 0.05$

Although total carotenoids reportedly (15) are stable in plasma for up to five days at room temperature, we found no reports that addressed the stability of individual carotenoids. Because products of carotenoid oxidation absorb light of similar wavelength, it is possible that the total may remain unchanged while a component is altered. Therefore, to confirm observations concerning the stability of retinol, tocopherol, and total carotenoids, and to determine the stability of individual carotenoids, we analyzed plasma left at room temperature for 0, 4, 12, and 24 h. There were no significant differences among the samples in the measured concentrations of retinol, tocopherol, or total or individual carotenoids. An apparent, but insignificant, reduction in lycopene was observed between 0 and 4 h.

Several reports (16, 18) cite increased stability of vitamin A and carotenoids when air is displaced from the tubes by an inert gas such as nitrogen. Unlike earlier reports, we did not observe a beneficial influence of purging samples with nitrogen. This observation may be ascribable to storage conditions whereby the samples were not freeze/thawed and headspace was small. Low-volume (1.8 mL), high-quality polypropylene vials with sealing, screw-top caps were used to store aliquots of plasma. Most previous investigations

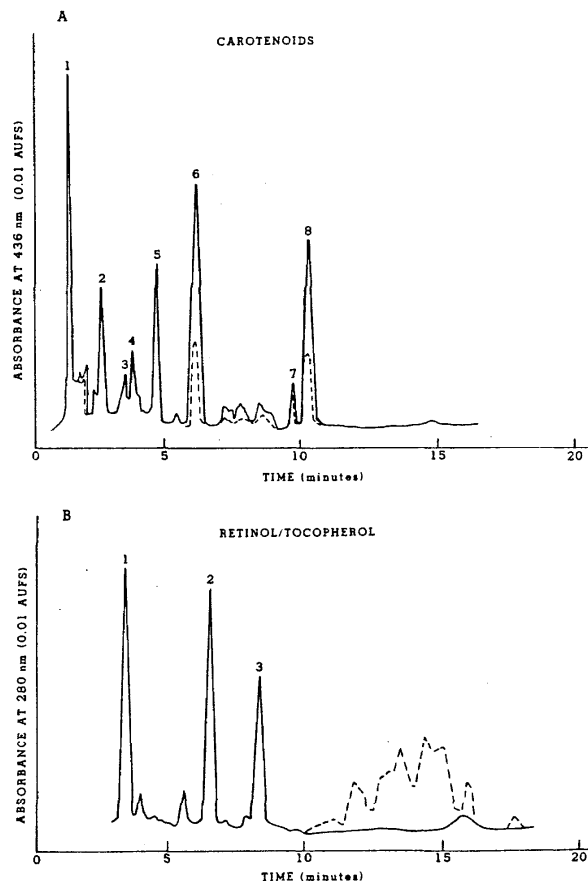


Fig. 5. Elution profile of carotenoids, retinol, and tocopherol in plasma samples stored for 15 months at either  $-70^{\circ}\text{C}$  (solid line) or  $-20^{\circ}\text{C}$  (dashed line)

A. Carotenoid profile ( $A_{436}$ ) illustrates loss of individual carotenenes. Peaks are designated as follows: 1, lutein and zeaxanthin; 2, unknown; 3, pre-cryptoxanthin; 4, cryptoxanthin; 5, internal standard (echinenone); 6, lycopene; 7, alpha-carotene; and 8, beta-carotene

B. Retinol/tocopherol profile ( $A_{280}$ ). Peaks are designated as follows: 1, retinol; 2, alpha-tocopherol; and 3, internal standard (alpha-tocopherol acetate). Note unidentified peaks eluted at 12 to 18 min in samples stored at  $-20^{\circ}\text{C}$

were not designed to study labile compounds and (or) did not have access to similar storage vials, thereby necessitating freeze/thawing and excessive exposure to oxygen. Under such conditions, nitrogen purging may substantially decrease oxidation.

With a view to automating the method, we tested the stability of analytes in organic solvents. Plasma extracts left at room temperature in the dark for as long as 18 h in appropriate solvents were found to be unchanged, a finding in agreement with studies by Matthews-Roth and Stampher (15) and Driskell et al. (3) in which total carotenoids determined spectrophotometrically and retinol/tocopherol determined by HPLC reportedly were stable, once extracted from serum. In contrast, the method of Peng et al. (8) is not suited for unattended mechanized analysis, because beta-carotene is only stable for 1 h in their solvent system.

Because the storage facilities used for many retrospective studies are often less than optimal, we investigated the effect of storage temperature on plasma retinol, tocopherol, and carotenoid concentrations. All analytes measured were stable for at least five months at either  $-70^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ . In agreement with the report of Driskell et al. (11), we ob-

served that retinol and tocopherol concentrations were not significantly changed in samples of plasma stored at  $-20^{\circ}\text{C}$  for 15 months. However, the concentrations of total and individual carotenoids decreased by 10 to 50%. This agrees with the observation of Matthews-Roth and Stampher (15), in which 97% loss of total carotene was reported in samples stored for 10 years at  $-20^{\circ}\text{C}$  and as much as 15% loss had occurred by six months.

The chromatographic profiles during the 12-min interval when analytes are eluted appeared unchanged for plasma samples at either storage temperature. However, for samples held at  $-20^{\circ}\text{C}$ , the appearance of several large peaks eluting after alpha-tocopherol acetate (12–18 min) with absorbance at 280 nm became evident after five months. Driskell et al. (12) reported oxidative degradation of analytes in stored plasma samples, which could be eliminated by the addition of 1 g of ascorbic acid per liter to the ethanol used for protein precipitation. Addition of neither ascorbic acid nor butylated hydroxytoluene altered the measured value for analytes or the presence of these unidentified peaks. Although their origin is unknown, one must be aware that these late-eluting peaks can interfere with subsequent injections.

In summary: we describe here a rapid, sensitive HPLC method of analysis for retinol, tocopherol, and individual carotenoids in plasma. Our work confirms the observation that retinol and tocopherol are stable for as long as 15 months when the plasma is stored at  $-20^{\circ}\text{C}$ . In contrast, there is significant carotenoid degradation by this time. Additionally, the individual carotenoids, retinol and tocopherol, when maintained at  $-70^{\circ}\text{C}$  under the given conditions, are stable for at least 28 months without nitrogen purging. The importance of this work is twofold: First, it challenges the validity of data generated by retrospective studies in which handling and (or) storage conditions were less than optimal. Secondly, we anticipate that the above information will assist in the design of prospective studies and clinical trials involving large numbers of plasma samples that will be analyzed for retinol, tocopherol, and carotenoids.

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